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published in

Arteriosclerosis, Thrombosis, and Vascular Biology

2001

DOI (link to publisher)

[10.1161/01.ATV.21.1.74](https://doi.org/10.1161/01.ATV.21.1.74)

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

Jager, A., Kostense, P. J., Nijpels, G., Dekker, J. M., Heine, R. J., Bouter, L. M., Donker, A. J. M., & Stehouwer, C. D. A. (2001). Serum homocysteine levels are associated with the development of (micro)albuminuria - The Hoorn Study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 21(1), 74-81.
<https://doi.org/10.1161/01.ATV.21.1.74>

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Serum Homocysteine Levels Are Associated With the Development of (Micro)albuminuria

The Hoorn Study

Agnes Jager, Piet J. Kostense, Giel Nijpels, Jacqueline M. Dekker, Robert J. Heine, Lex M. Bouter, Ab J.M. Donker, Coen D.A. Stehouwer

Abstract—Microalbuminuria is a strong indicator of the risk of future cardiovascular disease and renal dysfunction. Slightly increased levels of homocysteine, an independent risk factor for atherothrombotic disease, have recently been found to be associated with the presence of (micro)albuminuria. However, it is unknown whether increased homocysteine levels precede the occurrence of (micro)albuminuria. Normoalbuminuric subjects ($n=316$, 66 with non-insulin-dependent diabetes mellitus [NIDDM]) of an age-stratified, sex-stratified, and glucose tolerance-stratified sample of a population-based cohort study were investigated at baseline and after a mean follow-up duration of 6.1 years. Development of (micro)albuminuria was defined as a mean albumin-to-creatinine ratio >2.0 mg/mmol at the follow-up examination. The cumulative incidence of (micro)albuminuria was 14.0% (9.7 % to 18.3%) among nondiabetic subjects and 22.7% (12.9% to 32.5%) among NIDDM patients. Age-adjusted, sex-adjusted, and glucose tolerance status-adjusted logistic regression analyses showed development of (micro)albuminuria to be significantly associated with baseline homocysteine levels >19.0 $\mu\text{mol/L}$ compared with homocysteine levels <9.1 $\mu\text{mol/L}$ (odds ratio [OR] 5.1, 95% CI 1.1 to 23.0). For homocysteine levels of 9.1 to 14.0 $\mu\text{mol/L}$ and 14.1 to 19.0 $\mu\text{mol/L}$, the values were OR 1.2 (95% CI 0.5 to 3.0) and OR 1.8 (95% CI 0.6 to 5.3), respectively. Additional adjustment for baseline insulin resistance, blood pressure, body mass index, presence of cardiovascular disease and retinopathy, current smoking, or estimates of glomerular filtration rate did not materially affect the results. Substituting homocysteine levels as a continuous variable for categories of homocysteine levels showed that a 5- $\mu\text{mol/L}$ increase of the homocysteine level was associated with an increased risk of developing (micro)albuminuria (OR 1.38, 95% CI 0.97 to 1.95). Analyses performed in nondiabetic and diabetic subjects separately gave similar results among nondiabetic subjects. Among diabetic subjects, the association between homocysteine level and (micro)albuminuria could not be estimated, because there was an insufficient number of diabetic subjects with high homocysteine levels. Hyperhomocysteinemia is an independent determinant of the development of (micro)albuminuria among nondiabetic subjects, even after adjustment for estimates of glomerular filtration rate. We could neither confirm nor reject an association between homocysteine levels and the development of (micro)albuminuria among NIDDM subjects. These data suggest that homocysteine may play a pathophysiological role in the development of (micro)albuminuria. (*Arterioscler Thromb Vasc Biol.* 2001;21:74-81.)

Key Words: homocysteine ■ microalbuminuria ■ prospective studies ■ non-insulin-dependent diabetes mellitus

Microalbuminuria, a slightly increased urinary albumin excretion, is associated with an increased risk of cardiovascular morbidity and mortality among patients with non-insulin-dependent diabetes mellitus (NIDDM)¹ and among nondiabetic subjects.² Furthermore, the presence of microalbuminuria in diabetes identifies subjects at high risk of developing persistent proteinuria.^{1,3} Thus, microalbuminuria is a strong indicator of the risk of future cardiovascular disease and renal dysfunction. Therefore, elucidating the determinants of microalbuminuria is of great importance and

may lead to knowledge about the pathophysiological mechanisms necessary for therapeutic innovations.

Prospective observational studies, conducted mainly among diabetic subjects, have identified several determinants associated with the development of microalbuminuria, ie, age, male sex, blood pressure, poor glycemic control, smoking, retinopathy, and coronary heart disease.⁴⁻¹¹ Other determinants, such as insulin resistance (and related variables¹²⁻¹⁴) and increased protein intake,^{15,16} have been proposed on the basis of their cross-sectional associations with microalbuminuria.

Received January 25, 2000; revision accepted July 11, 2000.

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Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

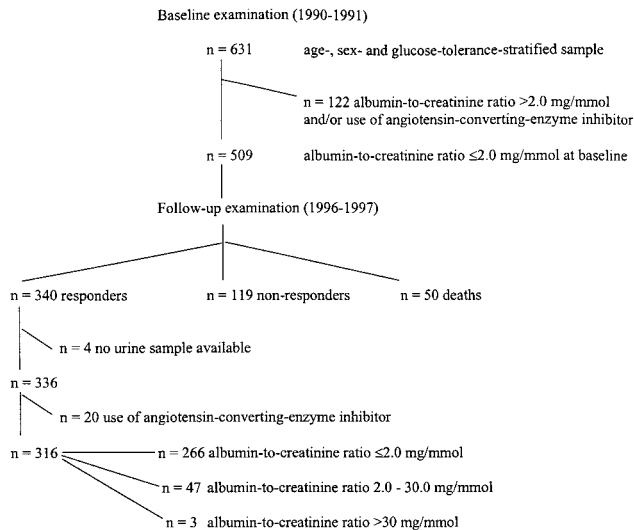


Figure 1. Study outline.

Interestingly, slightly increased levels of homocysteine, a sulfur-containing amino acid formed by demethylation of methionine, have recently been found to be associated with the presence of microalbuminuria,¹⁶⁻¹⁸ although not all studies concur.¹⁹ By definition, however, these cross-sectional investigations¹⁶⁻¹⁹ cannot establish whether increased homocysteine levels precede the occurrence of microalbuminuria, which would be an important step toward establishing increased homocysteine levels as a causal determinant of microalbuminuria.

Therefore, we performed a prospective study in an age-stratified, sex-stratified, and glucose tolerance-stratified sample of a population-based cohort study to investigate the association between homocysteine levels and the development of (micro)albuminuria. In addition, we gave particular attention to other proposed determinants for developing (micro)albuminuria, namely, high blood pressure, poor glycemic control, obesity, insulin resistance, a high protein intake, retinopathy, and cardiovascular disease.^{4-16,20}

Methods

General Study Design

The Hoorn Study is a cohort study of disturbances of glucose tolerance and cardiovascular disease in a general white population aged 50 to 75 years ($n=2484$). The baseline examinations were conducted from October 1989 until February 1992.^{21,22} For reasons of efficiency, we chose to study a smaller ($n=631$), but still randomly selected, sample in more detail with regard to cardiovascular disease. This sample was stratified for age, sex, and glucose tolerance status. We oversampled subjects with impaired glucose tolerance and NIDDM to increase the power of comparisons with subjects with normal glucose tolerance. Approximately 6 years after the baseline examination, from January 1996 until December 1997, all subjects were invited for a follow-up examination.

For the present analyses, we focused only on those subjects who, at baseline, were normoalbuminuric and did not use ACE inhibitors ($n=509$, 252 subjects with normal glucose tolerance, 136 subjects with impaired glucose tolerance, and 121 subjects with NIDDM). Between the baseline and the follow-up examinations, 50 (9.8%) subjects died, 32 (6.3%) moved out of Hoorn and were therefore not invited, and 87 (17.1%) did not respond to the invitation, leaving 340 subjects participating in the follow-up examination (Figure 1).

All participants gave informed consent for the baseline and follow-up examinations, which were approved by the local ethics committee.

Procedures at the Baseline Examination

An early morning first-voided spot urine sample was obtained, and the urinary albumin-to-creatinine ratio was determined. In a representative sample of 157 (31%) subjects, 2 early morning first-voided spot urine collections were available, and the presence of normoalbuminuria for these subjects was therefore based on the mean albumin-to-creatinine ratio of the 2 urine collections. Subjects were classified as having normoalbuminuria when they had a urinary albumin concentration below the assay threshold (6.2 mg/L, $n=321$) or a (mean) albumin-to-creatinine ratio ≤ 2.0 mg/mmol ($n=188$). (An overnight albumin-to-creatinine ratio >2.0 mg/mmol has a high sensitivity to detect an albumin excretion rate >30 $\mu\text{g}/\text{min}$.²³) The presence of leukocytes and nitrite was tested by dipstick.

We obtained data on blood pressure, weight, height, waist and hip circumference, fasting and 2-hour postload glucose, glycated hemoglobin, and serum fasting specific insulin, creatinine, homocysteine, urea nitrogen, albumin, total cholesterol, HDL cholesterol, and triglyceride levels.^{16,21,22} Hypertension was defined as diastolic pressure ≥ 95 mm Hg, systolic pressure ≥ 160 mm Hg, and/or the use of antihypertensive drugs.²¹ Insulin resistance was calculated by using the homeostasis model assessment formula (fasting insulin \times fasting glucose/22.4²⁴). The glomerular filtration rate (GFR) was estimated in 2 ways: (1) from the Cockcroft-Gault formula²⁵ for creatinine clearance ($n=340$, 100%) and (2) from the formula proposed by the Modification of Diet in Renal Disease Study Group (MDRD formula; $n=330$, 97.0%): $\text{GFR} = 170 \times (\text{serum creatinine level [mg/dL]})^{-0.726} \times (\text{age})^{-0.176} \times (0.762 \text{ if patients is female}) \times (1.180 \text{ if patient is black}) \times (\text{serum urea nitrogen level [mg/dL]})^{-0.170} \times (\text{serum albumin level [g/dL]})^{0.318}$.²⁶ Information about smoking habits and dietary protein intake was obtained with standardized questionnaires.²¹ Current smoking was defined as currently smoking cigarettes and/or cigars. We obtained an ankle-brachial blood pressure index and a resting ECG, as previously described.^{21,22} The cardiovascular and cerebrovascular history was obtained by means of the self-administered Rose questionnaire ($n=340$, 100%).²¹ Subjects were classified as having cardiovascular disease when they had an ankle-brachial pressure index <0.9 in either leg, when they had undergone a peripheral arterial bypass or amputation, when they had an ECG with a Minnesota code 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, or 7.1, when they had undergone coronary bypass surgery or angioplasty, and/or when they had self-reported myocardial infarction and/or cerebrovascular disease ($n=338$, 99.4%). Retinopathy was assessed by ophthalmoscopy ($n=337$, 99.1%) and/or fundus photography ($n=253$, 74.4%). (Fundus photographs of 87 [25.6%] subjects were lost. Loss was random with regard to age, sex, hypertension, and glucose tolerance categories [data not shown].) Both eyes were dilated, whereupon indirect and direct ophthalmoscopy was carried out by 1 of 2 ophthalmologists. Thereafter, 2 black and white photographs were taken of each eye, centered on the macular area and the optic disc. Both ophthalmoscopic and fundus photographic findings were independently graded by 2 ophthalmologists according to the modified Airlie House Classification.²⁷ In case of disagreement, the independent judgment of a third ophthalmologist was taken to be decisive. Retinopathy was defined as the presence of ≥ 1 hemorrhages, microaneurysms, soft and hard exudates, areas of neovascularization, and/or laser coagulation scars in at least 1 eye ($n=336$, 98.8%).

Procedures at the Follow-Up Examination

All participants were asked to hand in an overnight first-voided and an untimed spot urine sample. In a representative sample ($n=161$), subjects were asked to hand in a second set of urine samples within 4 weeks. Of all 340 participants, 4 collected no urine samples, 18 collected 1 sample, 168 collected 2 samples, 10 collected 3 samples, and 140 collected 4 samples. The urinary albumin-to-creatinine ratios were determined, and in case of >1 urine collection per participant, the mean albumin-to-creatinine ratio was calculated. Urinary albumin assessments below the threshold (2.0 mg/L) were set at 1.5 mg/L ($n=51$ urine samples among 38 subjects, 8% of all

urine samples). Subjects were classified as having normoalbuminuria when the mean albumin-to-creatinine ratio was ≤ 2.0 mg/mmol and as having (micro)albuminuria when the mean albumin-to-creatinine ratio was >2.0 mg/mmol. (An overnight albumin-to-creatinine ratio >2.0 mg/mmol has a high sensitivity to detect an albumin excretion rate >30 $\mu\text{g}/\text{min}$.²³) Subjects who used an ACE inhibitor ($n=20$) were excluded from further analyses (Figure 1). The presence of leukocytes and nitrite was tested by dipstick.

Assays

Urinary albumin was measured by rate nephelometry (Array Protein System, Beckman) with intra-assay and interassay coefficients of variation of $\leq 5\%$ and $\leq 8\%$, respectively (assay threshold was 6.2 mg/L at baseline and 2.0 mg/L at follow-up examination). Urinary and serum creatinine was measured by a modified Jaffe's test. Serum homocysteine concentrations were determined by high-performance liquid chromatography with fluorescence detection.¹⁶ Serum levels of urea nitrogen concentration were determined in samples stored at -70°C by a kinetic UV assay from Roche Diagnostics. Serum albumin levels were assessed by use of the bromocresol purple method. Levels of fasting and 2-hour postload venous plasma glucose, glycated hemoglobin, serum-specific fasting insulin, triglycerides, and total and HDL cholesterol were determined as previously described in detail.^{21,22}

Statistical Analyses

All analyses were performed with SPSS 7.5 for Windows 95. Differences between 2 groups in continuous variables that had a normal distribution were tested by Student *t* test; for continuous variables that had a skewed distribution, Mann-Whitney tests were used; and for the percentage of subjects with versus without the presence of dichotomous variables, the χ^2 test was used. To elucidate the associations of potential determinants with the development of (micro)albuminuria, logistic regression analyses were performed with the presence of (micro)albuminuria at follow-up as dependent variables and with potential determinants at baseline as independent variables. The Wald test was used to test significance. (Performing the analyses according to the log likelihood test gave similar results.) All ORs for developing (micro)albuminuria were adjusted for the original stratification variables: age, sex, and glucose tolerance status (ie, impaired glucose tolerance and NIDDM). Potential determinants measured on a continuous scale were used as such in the regression models, except for glycated hemoglobin, HDL cholesterol, body mass index, and protein intake, because the association of these variables with incident (micro)albuminuria was nonlinear. Therefore, a high glycated hemoglobin level was defined as a level $>6.5\%$; a low HDL cholesterol level was defined as a level <0.9 mmol/L²²; a high body mass index was defined as values >27 kg/m² for men and >26 kg/m² for women²²; and a high protein intake was defined as an intake >1.25 g/kg per day. Insulin resistance and fasting insulin and triglyceride levels were logarithmically transformed because of a better fit of the regression model. We evaluated a possible dose-response relation of homocysteine by calculating ORs for developing (micro)albuminuria for several ranges of homocysteine concentrations (9.1 to 14.0, 14.1 to 19.0, and >19.0 $\mu\text{mol}/\text{L}$) with homocysteine levels <9.1 $\mu\text{mol}/\text{L}$ as reference. By using this procedure, we evaded assumptions about linearity.^{16,28} To assess whether determinants were independently associated with development of (micro)albuminuria, regression analyses were primarily adjusted for all variables that were statistically significant in the initial analyses and secondarily for other potential variables of interest. All these variables were entered simultaneously into the regression models. Two-sided values of $P<0.05$ were considered statistically significant.

Results

The mean duration of follow-up was 6.1 years (SD 0.7 years, range 4.4 to 7.7 years). The cumulative incidence of (micro)albuminuria at 6.1 years of follow-up was 14.0% (95% CI 9.7 to 19.3) among nondiabetic subjects and 22.7% (95% CI 12.9 to 32.5) among NIDDM patients. The cumulative

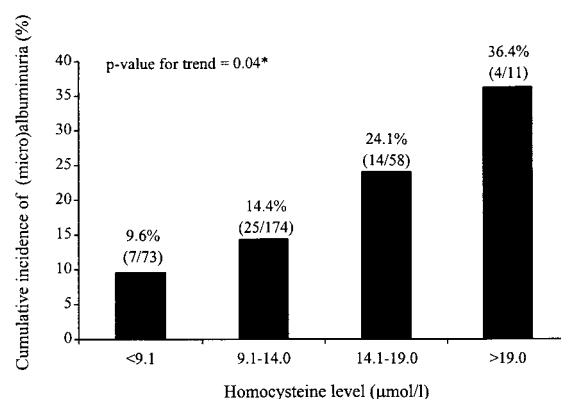


Figure 2. Cumulative incidence of (micro)albuminuria per category of homocysteine level. *Estimated by age-adjusted, sex-adjusted, and glucose tolerance-adjusted logistic regression analyses.

incidence of (micro)albuminuria increased with categories of increasing homocysteine levels (Figure 2).

Compared with subjects who participated in the follow-up examination, subjects who died during the follow-up period were older, more insulin resistant, more obese, and more often smokers, and they more often had NIDDM, hypertension, cardiovascular disease, higher levels of homocysteine and triglycerides, and a lower protein intake at baseline (data not shown). The nonresponders (Figure 1) were not materially different from those who did participate in the following examination with regard to the variables shown in Table 1, except that nonresponders were older (65 versus 63 years) and had higher levels of homocysteine (13.1 versus 11.8 $\mu\text{mol}/\text{L}$).

Compared with subjects who did not develop (micro)albuminuria, subjects who developed (micro)albuminuria were significantly older, more insulin resistant, and more obese; they had higher blood pressures and higher fasting insulin and homocysteine levels; and they more often had hypertension, cardiovascular disease, and retinopathy at baseline (Table 1). Age-adjusted, sex-adjusted, and glucose tolerance-adjusted logistic regression analyses showed the development of (micro)albuminuria to be significantly associated with age, male sex, fasting insulin levels, insulin resistance, systolic and diastolic blood pressure, the presence of hypertension, body mass index, homocysteine levels, and the presence of cardiovascular disease (Table 2). Analyses performed in nondiabetic and diabetic subjects separately gave similar results, except for the association between the development of (micro)albuminuria on the one hand and glycated hemoglobin and homocysteine levels on the other, which could be estimated only among nondiabetic subjects (Table 2), the latter because there was only 1 diabetic subject with homocysteine levels >19.0 $\mu\text{mol}/\text{L}$.

To further investigate the association between development of (micro)albuminuria and potential determinants, multiple regression analyses were performed with all variables significantly associated with the development of (micro)albuminuria (Table 2), as well as the stratification variables, as independent variables. These adjustments did not materially affect the results, except for the association between the development of (micro)albuminuria and fasting insulin levels, which was no longer statistically significant (Table 3).

TABLE 1. Baseline Characteristics According to Albuminuria Status at Follow-Up

	Persistent Normoalbuminuria (n=266)	Development of (Micro)albuminuria (n=50)
Male/female, n/n	128/138	30/20
Age, y	62±7	66±7¶
Fasting glucose, mmol/L	6.2±1.8	6.6±1.9
Glycated hemoglobin, %	5.6±1.0	5.7±1.0
Fasting insulin,* pmol/L	79 (60–107)	103 (75–138)¶
Insulin resistance (dimensionless)*†	20.4 (14.5–30.7)	27.3 (19.5–43.1)¶
Impaired glucose tolerance, %	26	32
NIDDM, %	19	30
Diastolic blood pressure, mm Hg	81±9	86±9¶
Systolic blood pressure, mm Hg	133±16	145±18¶
Hypertension, %	23	56¶
Waist-to-hip ratio (men)	0.96±0.06	0.97±0.07
Waist-to-hip ratio (women)	0.86±0.08	0.90±0.07¶
Body mass index, kg/m ²	26.4±3.4	27.8±3.7¶
Total cholesterol, mmol/L	6.6±1.1	6.5±1.4
HDL cholesterol, mmol/L	1.3±0.4	1.2±0.3
Triglycerides,* mmol/L	1.5 (1.0–2.1)	1.5 (1.2–2.3)
Homocysteine, μmol/L	11.6±4.3	13.0±4.0¶
Total protein intake, g · kg ⁻¹ · d ⁻¹	1.0±0.3	1.0±0.3
Animal protein intake, g · kg ⁻¹ · d ⁻¹	0.7±0.2	0.7±0.2
Cardiovascular disease,‡ %	14	34¶
Retinopathy, %	8	18¶
Current smoking, %	28	20
Creatinine clearance,§ mL/min	80±17	78±18
Range	42–150	42–120
GFR, mL · min ⁻¹ · 1.73 m ⁻²	70±11	68±12
Range	44–102	43–100

Data are means±SD unless otherwise indicated.

*Median (25–75th percentiles).

†Calculated according to homeostasis model assessment formula (fasting insulin×fasting glucose/22.4).²³

‡Ankle-brachial pressure index <0.90 and/or peripheral bypass operation, amputation and/or Minnesota code 1.1–1.3, 4.1–4.3, 5.1–5.3, or 7.1 on ECG, coronary bypass operation or angioplasty, and/or self-reported myocardial infarction or cerebrovascular disease.

§Calculated by Cockcroft-Gault formula.²⁴||Calculated according to MDRD formula.²⁵

¶P<0.05 vs persistent normoalbuminuria.

Further adjustment for creatinine clearance did not materially change the association between homocysteine level and the development of (micro)albuminuria (eg, among all subjects, the ORs were 1.4 [95% CI 0.5 to 3.8], 1.7 [95% CI 0.5 to 5.5], and 7.6 [95% CI 1.5 to 39.3] for homocysteine levels of 9.1 to 14.0, 14.1 to 19.0, and >19.0 μmol/L compared with homocysteine levels <9.1 μmol/L, respectively). Substituting GFR estimated from the MDRD formula for creatinine clearance again did not materially change the association between homocysteine level and the development of (micro)albuminuria (eg, among all subjects, the ORs were 1.4 [95% CI 0.5 to 3.9], 1.7 [95% CI 0.5 to 5.5], and 8.4 [95% CI

1.6 to 44.8] for homocysteine levels of 9.1 to 14.0, 14.1 to 19.0, and >19.0 μmol/L compared with homocysteine levels of <9.1 μmol/L, respectively). Additional adjustment for retinopathy and current smoking also did not materially change the results (data not shown). Substituting insulin resistance for fasting insulin levels or systolic blood pressure or hypertension for diastolic blood pressure again did not materially change the results (data not shown). Changing the cutoff levels of categories of homocysteine levels (<10.1 [reference category], 10.1 to 14.0, 14.1 to 18.0, and >18.0 μmol/L) somewhat decreased the ORs for developing (micro)albuminuria (eg, among all subjects, 1.0 [reference category], 1.0 [95% CI 0.4 to 2.2], 1.3 [95% CI 0.5 to 3.5], and 4.8 [95% CI 1.3 to 18.0], respectively, after adjusting for the determinants shown in Table 3). Substituting homocysteine levels as continuous variables for categories of homocysteine levels showed that a 5-μmol/L increase of the homocysteine level was associated with a 1.4 (95% CI 0.97 to 2.0) increased risk of developing (micro)albuminuria after adjusting for the determinants shown in Table 3.

Among all subjects, the ORs of developing (micro)albuminuria after adjustment for the determinants shown in Table 3 were 2.0 (95% CI 0.7 to 5.6) for total protein intake (>1.25 g/kg per day versus ≤1.25 g/kg per day) and 2.7 (95% CI 1.0 to 7.3) for retinopathy (yes versus no).

Additional Analyses

The following additional analyses did not materially affect our results: analyses with (micro)albuminuria defined as an albumin-to-creatinine ratio >3.0 instead of >2.0 mg/mmol, analyses with (micro) albuminuria defined on the basis of first-voided overnight urine or untimed spot urine samples only, analyses with (micro)albuminuria defined on the basis of the median albumin-to-creatinine ratio, analyses after exclusion of subjects who developed macroalbuminuria (as defined by an albumin-to-creatinine ratio >30 mg/mmol, n=3), analyses after exclusion of urine samples with a positive dipstick test for leukocytes and/or nitrite at baseline (n=49) and/or at follow-up (n=47) measurement, and (among nondiabetic subjects) analyses that excluded subjects with impaired glucose tolerance (n=86, data not shown).

Discussion

In the present prospective study, we showed that hyperhomocysteinemia is an independent determinant of the development of (micro)albuminuria among nondiabetic subjects, even after adjustment for 2 different estimates of the glomerular filtration rate.^{24,25} We could neither confirm nor reject an association between homocysteine and the development of (micro)albuminuria among NIDDM subjects. These data are in line with previous cross-sectional findings^{16–18} and support the hypothesis that homocysteine may play a pathophysiological role in the development of (micro)albuminuria.

This is the first study showing that an increased homocysteine level precedes the development of (micro)albuminuria. This is an important step toward establishing high homocysteine levels as a causal risk factor for (micro)albuminuria, as previously suggested by cross-sectional studies.^{16–18} We found a possible threshold of homocysteine level above which the risk of (micro)albuminuria increased at ≈19 μmol/L, which is similar to the threshold of 18 μmol/L found

TABLE 2. Age-Adjusted, Sex-Adjusted, and Glucose Tolerance-Adjusted ORs for Developing (Micro)albuminuria of Potential Determinants

	All Subjects (n=316), OR (95% CI)*	Nondiabetic Subjects (n=250), OR (95% CI)*	Diabetic Subjects (n=66), OR (95% CI)*
Age (per 5-year increase)	1.5 (1.2–1.9)	1.4 (1.1–1.8)	1.9 (1.1–3.2)
Male (vs female)	2.0 (1.0–3.7)	1.6 (0.8–3.3)	3.6 (0.98–13.2)
Fasting insulin (per 10% increase)	1.1 (1.0–1.2)	1.1 (0.99–1.2)	1.2 (0.98–1.4)
Insulin resistance (per 10% increase)‡	1.1 (1.0–1.2)	1.1 (1.0–1.2)	1.1 (0.96–1.3)
Glycated hemoglobin (> vs ≤6.0%)	1.4 (0.6–3.2)	1.6 (0.5–5.1)	1.0 (0.3–3.8)
Impaired glucose tolerance (vs NGT)	1.5 (0.7–3.2)	1.6 (0.7–3.3)	...
NIDDM (vs NGT)	2.1 (0.96–4.5)
DBP (per 10 mm Hg increase)	1.9 (1.4–2.8)	2.0 (1.3–2.9)	2.3 (1.0–5.3)
SBP (per 10 mm Hg increase)	1.4 (1.1–1.7)	1.5 (1.2–1.8)	1.2 (0.8–1.8)
Hypertension (yes vs no)	3.8 (2.0–7.5)	3.8 (1.7–8.2)	4.6 (1.1–18.7)
Waist-to-hip ratio (per 0.01 increase)	1.0 (0.98–1.1)	1.0 (0.99–1.1)	1.0 (0.9–1.1)
Body mass index (yes vs no)§	3.1 (1.5–6.3)	2.6 (1.2–5.7)	5.9 (1.0–33.7)
Total cholesterol (per 1-mmol/L increase)	1.0 (0.8–1.3)	1.1 (0.8–1.6)	0.8 (0.4–1.4)
HDL cholesterol (≤ vs >0.9 mmol/L)	1.4 (0.5–3.7)	2.4 (0.7–7.7)	0.4 (0.1–2.8)
Triglycerides (per 10% increase)	1.0 (0.9–1.1)	1.0 (0.9–1.1)	1.0 (0.9–1.1)
Homocysteine			
9.1–14.0 vs <9.1 μmol/L†	1.2 (0.5–3.0)	1.2 (0.4–3.6)	1.0 (0.2–6.4)
14.1–19.0 vs <9.1 μmol/L†	1.8 (0.6–5.3)	1.4 (0.4–5.0)	3.6 (0.5–28.4)
>19.0 vs <9.1 μmol/L†	5.1 (1.1–23.0)	5.6 (1.1–27.6)	...¶
Homocysteine (per 5-μmol/L increase)	1.3 (0.9–1.8)	1.2 (0.9–1.8)	1.6 (0.6–4.5)
Total protein intake (> vs ≤1.25 g · kg ⁻¹ · d ⁻¹)	1.4 (0.6–3.3)	1.7 (0.6–4.7)	0.8 (0.1–4.6)
Cardiovascular disease (yes vs no)‡	2.2 (1.1–4.3)	2.4 (1.1–5.3)	1.6 (0.4–6.2)
Retinopathy (yes vs no)	2.3 (0.96–5.7)	2.2 (0.8–5.8)	3.7 (0.4–36.2)
Creatinine clearance			
51–80 vs >80 mL/min‡	1.1 (0.5–2.8)	0.8 (0.3–1.9)	2.01 (0.46–8.83)
<51 vs >80 mL/min‡	2.43 (0.55–10.78)	1.60 (0.31–8.30)	...#
GFR (per 10-mL · min ⁻¹ · 1.73 m ⁻² decrease)‡	1.23 (0.90–1.67)	0.95 (0.73–1.49)	1.79 (0.94–3.45)

NGT indicates normal glucose tolerance; DBP, diastolic blood pressure; and SBP, systolic blood pressure.

*OR and 95% CI were performed by logistic regression analyses with development of (micro)albuminuria as dependent variable.

†Among nondiabetic subjects, the numbers of subjects with microalbuminuria per increasing category of homocysteine levels were 5 of 56, 18 of 137, 8 of 47, and 4 of 10, respectively. Among diabetic subjects, the numbers of subjects with microalbuminuria per increasing category of homocysteine levels were 2 of 17, 7 of 37, 6 of 11, and 0 of 1, respectively.

‡Described in legend to Table 1.

§>27 vs ≤27 kg/m² for men and >26 vs ≤26 kg/m² for women.

||*P*<0.05 for trend of the homocysteine categories.

¶One subject with homocysteine levels >19.0 μmol/L.

#Only 3 subjects with creatinine clearance <51 mL/min.

in our previous cross-sectional study.¹⁶ Clearly, the present study is too small to establish with certainty the existence and the exact level of such a threshold.

The pathophysiological pathway linking homocysteine level and risk of (micro)albuminuria is unknown. Some evidence suggests that hyperhomocysteinemia enhances oxidative stress, which could induce endothelial and mesangial cell dysfunction.²⁹ Intact renal endothelial and mesangial cell function is important for regulating intraglomerular pressure and glomerular charge and size selectivity. Dysfunction of these cells may increase intraglomerular pressure and/or

decrease glomerular charge and size selectivity and thus cause microalbuminuria. Alternatively, hyperhomocysteinemia and (micro)albuminuria could be associated through a common pathophysiological pathway; eg, inadequate vitamin B₆, B₁₂, and/or folate status could be the common antecedent leading to hyperhomocysteinemia on the one hand and to the development of (micro)albuminuria on the other. However, there is no evidence that inadequate B-vitamin status can directly cause (micro)albuminuria. Finally, hyperhomocysteinemia could be indirectly related to (micro)albuminuria; ie, hyperhomocysteinemia may influence another factor, such as

TABLE 3. Multiple Logistic Regression Analyses With Development of (Micro)albuminuria as Dependent Variable

	All Subjects (n=316), OR (95% CI)*	Nondiabetic Subjects (n=250), OR (95% CI)*	Diabetic Subjects (n=66), OR (95% CI)*
Age (per 5-year increase)	1.5 (1.1–1.9)	1.3 (0.97–1.8)	2.5 (1.2–5.2)
Male (vs female)	1.9 (0.9–3.8)	1.5 (0.6–3.4)	4.4 (0.9–21.7)
Fasting insulin (per 10% increase)†	1.0 (0.96–1.1)	1.0 (0.95–1.1)	1.1 (0.9–1.3)
Impaired glucose tolerance (vs NGT)	1.1 (0.5–2.5)	1.1 (0.5–2.5)	...
NIDDM (yes vs no)	1.4 (0.6–3.3)
DBP (per 10 mm Hg increase)†	1.9 (1.3–2.8)	1.9 (1.2–3.0)	3.1 (1.0–9.4)
Body mass index (yes vs no)‡	2.6 (1.2–5.8)	2.3 (0.9–5.6)	2.8 (0.4–20.0)
Cardiovascular disease (yes vs no)§	2.4 (1.2–5.0)	2.7 (1.1–6.4)	2.3 (0.4–12.5)
Homocysteine			
9.1–14.0 vs <9.1 $\mu\text{mol/L}$	1.4 (0.5–3.8)	1.7 (0.5–5.3)	0.6 (0.1–5.5)
14.1–19.0 vs <9.1 $\mu\text{mol/L}$	1.9 (0.6–5.7)	1.6 (0.4–6.0)	1.9 (0.2–19.0)
>19.0 vs <9.1 $\mu\text{mol/L}$	8.5 (1.7–42.6)	12.2 (2.1–69.8)	.. ‡

Variables included in this analysis were those that were significantly ($P<0.05$) associated with the development of microalbuminuria in initial analyses among all subjects (Table 2, first column). The variables “impaired glucose tolerance” and “NIDDM” were also included in the model, because they were stratification variables.

*OR (95% CI) of developing microalbuminuria obtained by logistic regression analyses.

†Substituting insulin resistance for fasting insulin levels or SBP or hypertension for DBP did not materially change the result.

‡Described in Table 2.

§Described in Table 1.

|| $P<0.05$ for trend of the homocysteine categories.

blood pressure, which directly causes the development of (micro)albuminuria. We found no evidence that this was the case. Nevertheless, we clearly cannot rule out these possibilities, and experimental work will be necessary to establish whether the association between homocysteine and microalbuminuria is causal.

Was confounding by variables other than vitamin status sufficiently excluded in our analyses? We adjusted for important variables known to affect homocysteine levels, such as sex, impaired renal function, and smoking habits, so it is unlikely that these factors confounded our results.

Follow-up was not complete (Figure 1), and it is important to consider whether this may have biased the association between homocysteine level and risk of incident (micro)albuminuria. First, 50 of the 509 subjects investigated at baseline died during follow-up. We have previously shown in this population that mortality risk is related to having NIDDM,²² (micro)albuminuria,²² and high homocysteine levels, especially in the presence of NIDDM,³⁰ and that high homocysteine levels and microalbuminuria are associated in cross-sectional analyses.¹⁶ Indeed, subjects who died had higher homocysteine levels at baseline than did those who survived. Taken together, these data suggest that compared with subjects who survived, subjects who died may have been at increased risk of developing (micro)albuminuria; this would result in an underestimation of the association between high homocysteine levels and the risk of (micro)albuminuria, in particular among diabetic subjects. Second, 119 nonresponders and 20 subjects who used ACE inhibitors were excluded from the analyses. Given their clinical profile, these subjects may have been at increased risk of developing

(micro)albuminuria compared with the participants, but again it is not clear why this should have led to an overestimation of the relation between homocysteine levels and the risk of (micro)albuminuria. Therefore, we conclude that, if anything, the incomplete follow-up may have resulted in an underestimation of the association between high homocysteine levels and incident (micro)albuminuria.

In the present study, the cumulative incidence of (micro)albuminuria after 6.1 years of follow-up was 14.0% among nondiabetic subjects and 22.7% among NIDDM patients, which is in agreement with previous reports.^{4,7,10,11} Risk of developing (micro)albuminuria was associated not only with high homocysteine levels but also with age, blood pressure, body mass index, and the presence of cardiovascular disease and retinopathy. The association with age is in accordance with other studies¹⁰ but is poorly understood mechanistically. Hypertension may increase intraglomerular pressure³¹ and thus promote albumin leakage,^{5,8,9} although prospective studies do not show consistent results.^{6,7,10,11} Obesity may increase the risk of microalbuminuria by altering intraglomerular hemodynamics.¹⁴ However, the association between obesity and microalbuminuria again is not consistently observed.^{5,9,10}

The link between retinopathy and (micro)albuminuria observed in the present and in previous prospective^{8,10} studies can probably be understood by considering that both reflect microangiopathy and thus share certain pathogenetic mechanisms, such as endothelial dysfunction. Endothelial dysfunction may also explain why the presence of cardiovascular disease confers an increased risk of incident microalbuminuria¹¹ as well as the converse.³²

Hyperglycemia can increase intraglomerular pressure and induce the loss of negative charges on the glomerular basement membrane, resulting in enhanced renal albumin leakage.^{31,33} Moreover, poor glycemic control has been shown to precede the development of microalbuminuria in diabetic subjects.^{6,8,10,11} Nevertheless, in the present study, we could not demonstrate glycemic control or the presence of NIDDM to be associated with incident (micro)albuminuria. The number of NIDDM subjects in the present study might have been too small; ie, there was a lack of power. Alternatively, selective mortality or selective improvement of glycemic control during follow-up among NIDDM subjects with a high, compared with those with a low, glycosylated hemoglobin level might explain the present findings.

Microalbuminuria has been proposed to be part of the insulin resistance syndrome,^{12,13} but not all studies concur.^{20,34,35} Previous prospective studies⁴ have not demonstrated a high insulin level or insulin resistance to be associated with an increased risk of developing microalbuminuria, which is in accordance with the present data.

Protein intake was not clearly associated with the risk of incident (micro)albuminuria, which contrasts somewhat with our previous cross-sectional finding.¹⁶ We assessed protein intake at baseline only. This may have been a poor estimate of protein intake during follow-up, inasmuch as dietary changes are likely, which would dilute any "true" association between protein intake and the risk of incident (micro)albuminuria.

The main limitation of the present study is the small number of subjects, resulting in risk estimates with large CIs, ie, substantial uncertainty. On the other hand, the main findings seem quite robust, inasmuch as adjustment for potential determinants did not materially change these findings.

In conclusion, we have shown that a high homocysteine level is independently associated with an increased risk of developing (micro)albuminuria. This finding supports the hypothesis that hyperhomocysteinemia may be a causal determinant of (micro)albuminuria, although further mechanistic work is needed to prove this hypothesis. Nevertheless, our findings could be of clinical relevance, because increased levels of homocysteine can be effectively treated with folic acid, vitamin B₆, and/or vitamin B₁₂.^{36,37}

Acknowledgments

This study was supported by a Clinical Research Fellowship from the Diabetes Fonds Nederland and the Netherlands Organization for Scientific Research (NOW) to C.D.A. Stehouwer.

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